The active molecular form of plasma adrenomedullin is extracted in the pulmonary circulation in patients with mitral stenosis: possible role of adrenomedullin in pulmonary hypertension

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**ABSTRACT**

Adrenomedullin (AM), a novel hypotensive peptide, preferentially dilates pulmonary vessels rather than systemic vessels. This suggests the possibility that AM is a circulating hormone which participates in regulation of the pulmonary circulation. A recent study revealed that two molecular forms of AM, i.e. a mature, active form of AM (AM-m) and an intermediate, inactive, glycine-extended form of AM (AM-Gly), circulate in human plasma. In the present study we investigated the production and clearance sites and pathophysiological significance of the two molecular forms of AM in the pulmonary circulation in patients with mitral stenosis. We measured the plasma levels of AM-m and total AM (AM-T; AM-m + AM-Gly) using a recently developed specific immunoradiometric assay, and thus calculated plasma AM-Gly levels, in blood samples obtained from the femoral vein, pulmonary artery, left atrium and aorta of 28 consecutive patients with mitral stenosis (20 females and eight males; age 53 ± 10 years). Patients with mitral stenosis had significantly higher venous concentrations of AM-T, AM-Gly and AM-m than age-matched normal controls (AM-T, 15.9 ± 2.5 and 10.6 ± 2.1 pmol/l respectively; AM-Gly, 14.0 ± 2.1 and 9.8 ± 1.9 pmol/l respectively; AM-m, 1.9 ± 0.6 and 1.1 ± 0.3 pmol/l respectively; each P < 0.001). There was a significant decrease in the concentrations of AM-m and AM-T between the pulmonary artery and the left atrium (AM-T, 16.1 ± 2.7 and 14.0 ± 2.4 pmol/l respectively; AM-m, 2.0 ± 0.6 and 0.7 ± 0.2 pmol/l respectively; each P < 0.001); however, there were no differences in plasma AM-Gly levels between the pulmonary artery and the left atrium (14.1 ± 2.3 and 13.5 ± 2.3 pmol/l respectively). The venous concentrations of AM-m, AM-Gly and AM-T showed similar correlations with mean pulmonary artery pressure (AM-T, r = 0.67; AM-Gly, r = 0.63; AM-m, r = 0.59; each P < 0.001) and total pulmonary vascular resistance (AM-T, r = 0.77; AM-Gly, r = 0.70; AM-m, r = 0.75; each P < 0.001). These results suggest that the plasma concentration of AM-m is increased in parallel with those of AM-Gly and AM-T, and that the main site for clearance of AM-m from the plasma is the lung; the extracted AM-m in the lungs may help to attenuate the increased pulmonary arterial resistance in secondary pulmonary hypertension due to mitral stenosis.

**Key words:** adrenomedullin, mitral stenosis, pulmonary circulation, pulmonary hypertension.

**Abbreviations:** AM, adrenomedullin; AM-Gly, glycine-extended form of AM; AM-m, mature, active form of AM; AM-T, total AM; ANP, atrial natriuretic peptide; IRMA, immunoradiometric assay; PTMC, percutaneous transmural commissurotomy.

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INTRODUCTION

Adrenomedullin (AM) is a hypotensive 52-amino-acid peptide that was originally discovered in acid extracts from human pheochromocytoma [1]. AM mRNA is reported to be highly expressed in the adrenal gland, lung, kidney, heart and vascular walls [1–3]. On the other hand, AM binding sites are highly expressed in rat lung [4]. A recent study demonstrated that AM receptor mRNA is abundantly expressed in human lung tissue [5]. AM has been shown to preferentially dilate the pulmonary vessels and increase pulmonary blood flow [6,7]. In addition, high plasma AM levels have been reported in rats with experimentally induced pulmonary hypertension and in patients with primary and secondary pulmonary hypertension [8–10]. These findings suggest the possibility that circulating AM may participate in the control of pulmonary vascular tone and in the pathophysiology of pulmonary hypertension.

The human AM precursor consists of 185 amino acids, including a putative signal peptide [11]. AM is produced from the AM precursor by a two-step enzymic reaction. First the AM precursor is converted into C-terminally glycine-extended AM (AM-Gly), a 53-amino-acid peptide that represents an intermediate, inactive form of AM. Subsequently, inactive AM-Gly is converted into the mature, active form of AM (AM-m), a 52-amino-acid peptide with a C-terminal amide structure, by enzymic amidation. Recently, Kitamura et al. [12] reported that both AM-m and AM-Gly circulate in human blood. However, the ratio of these two molecular forms of plasma AM, as well as their production and clearance sites and their pathophysiological significance in patients with mitral stenosis with pulmonary hypertension, remains unknown.

The present study aimed to explore the production and clearance sites and the pathophysiological significance of these two molecular forms of AM in patients with mitral stenosis with pulmonary hypertension. We measured plasma AM-m and AM-Gly concentrations in blood samples obtained from the femoral vein, pulmonary artery, left atrium and aorta of 28 consecutive patients with mitral stenosis, using a recently developed specific immunoradiometric assay (IRMA) [13,14].

METHODS

Patients

A total of 28 consecutive symptomatic Japanese patients with mitral stenosis (20 females and eight males; age 33–70 years; mean age 53 ± 10 years), who underwent percutaneous transmitral commissurotomy (PTMC), were enrolled in the study. Twenty of the patients had atrial fibrillation, and eight had sinus rhythm. Seven patients had a history of right-sided heart failure. All the patients underwent trans-thoracic echocardiograms, and their left ventricular function was normal. Informed consent was obtained from each patient. The patients' characteristics are presented in Table 1. The patients with mitral stenosis had higher plasma atrial natriuretic peptide (ANP) levels and a lower body mass index than the normal controls, although there were no significant differences in serum creatinine levels between the two groups. All the patients had trans-oesophageal echocardiograms, and patients with left atrial thrombus and those with severe mitral regurgitation (greater than 2/4 grade) were excluded from study, as were patients with diabetes mellitus, chronic renal failure or hepatic insufficiency.

Age- and sex-matched normotensive subjects (n = 55; 26 males and 29 females; mean age 52 ± 7 years) who entered a 2-day hospitalized health check programme in our institute served as controls. Characteristics of the normal controls are also presented in Table 1. Subjects found during the comprehensive check-up to be suffering from hypertension, cardiovascular disease, or renal, hepatic, metabolic or endocrine disease were excluded. All controls were hospitalized and given a diet containing 120–170 mmol of sodium. Informed consent was obtained from the participants prior to initiation of the study. Approval for the study was granted by the hospital ethical committee.

Cardiac catheterization

All patients underwent right- and left-sided heart catheterization before PTMC. PTMC was performed with an Inoue balloon catheter by the previously de-
scribed trans-septal Brockenbrough method [15–17]. Haemodynamic variables, including pressure in the pulmonary artery, left atrium, left ventricle and aorta, and the cardiac index, were measured. Left atrial pressure and left ventricular pressure were recorded simultaneously to determine the mean transmitral gradient. The cardiac output was determined by the thermodilution method. Systemic vascular resistance, pulmonary arterial resistance and total pulmonary resistance were determined using the standard formulae. The mitral valve area was calculated using Gorlin’s formula.

**Blood sampling**

Blood samples were obtained via catheters from the femoral vein, pulmonary artery, left atrium and aorta of patients with mitral stenosis. For normal controls, blood samples were obtained through the antecubital vein. The blood was transferred immediately into a chilled glass tube containing disodium EDTA (1 mg/ml) and apro tinin (500 units/ml) for the measurement of plasma concentrations of AM-m, total AM (AM-T; AM-m + AM-Gly) and ANP. The blood was centrifuged immediately at 4 °C, and the plasma was frozen and stored at −80 °C until assayed.

**Assays**

Both AM-m and AM-T were measured using recently developed specific IRMA kits (AM mature RIA and AM RIA; Shionogi Co., Osaka, Japan) [13,14]. These assay systems use two monoclonal antibodies against human AM, one recognizing a ring structure of human AM (both kits) and the other recognizing either the C-terminal sequence (AM-m kit) or AM-(25–36) (AM-T kit). The assay measures human AM-m or AM-T by sandwiching it between the two antibodies without the extraction of plasma. The minimum quantity of human AM-m or AM-T detectable using these assays is 0.5 pmol/l (both kits). The plasma AM-Gly concentration was calculated using the following formula: AM-Gly = AM-T – AM-m. Plasma concentrations of ANP were measured using the Shiono RIA ANP assay kit (Shionogi Co.), as reported previously [18]. Plasma creatinine was measured colorimetrically by the ion-selective method.

**Statistical analysis**

All data are expressed as means ± S.D. Student’s unpaired t-test or the χ² test was used to evaluate differences between the normal controls and the patients with mitral stenosis. The comparisons of plasma AM concentrations between the vein, pulmonary artery, left atrium and aorta were done by nested ANOVA followed by Scheffe’s test. The correlation coefficients were calculated by linear regression analysis. P values of < 0.05 were considered significant.

**RESULTS**

**Haemodynamic characteristics and plasma AM concentrations**

Haemodynamic variables in patients with mitral stenosis are presented in Table 2. These patients with mitral stenosis are characterized by higher mean pulmonary arterial pressure, left atrial pressure, total pulmonary resistance, pulmonary vascular resistance and plasma ANP concentration, and a lower mitral valve area.

The venous plasma concentrations of AM-m, AM-Gly and AM-T in the normal control subjects and in the patients with mitral stenosis are shown in Figure 1. Venous plasma AM-m, AM-Gly and AM-T levels in the patients were higher than those in the age-matched normal controls; the extent of the increase was similar for all forms (all P < 0.001). The relationship between the concentrations of AM-m and AM-T is shown in Figure 2. There was a good relationship between the levels of AM-m and AM-T (r = 0.78, P < 0.001).

There were no differences in the concentrations of AM-T and AM-m between the femoral vein and the pulmonary artery or between the left atrium and the aorta (Table 3). However, the concentrations of AM-T and AM-m were significantly lowered in the left atrium and aorta compared with the pulmonary artery and pulmonary vein (Table 3). This decrease in AM levels between the left atrium and the pulmonary artery was apparently greater for AM-m than for AM-T. In contrast, there were no differences in plasma AM-Gly levels between any of the sample sites (Table 3). These findings indicate that the decrease in AM-T levels between the pulmonary artery and the left atrium are due mainly to a decrease in AM-m.

**Relationships of plasma AM-m, AM-Gly and AM-T levels with haemodynamic variables**

Correlations between the venous concentrations of AM-m, AM-Gly and AM-T and haemodynamic variables are presented in Table 2. These patients with mitral stenosis are characterized by higher mean pulmonary arterial pressure, left atrial pressure, total pulmonary resistance, pulmonary vascular resistance and plasma ANP concentration, and a lower mitral valve area.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Mean pulmonary artery pressure (mmHg)</td>
<td>24.7 ± 7.0</td>
</tr>
<tr>
<td>Left atrial pressure (mmHg)</td>
<td>16.0 ± 5.0</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>8.3 ± 3.3</td>
</tr>
<tr>
<td>Mitral valve area (cm²)</td>
<td>1.40 ± 0.34</td>
</tr>
<tr>
<td>Total pulmonary resistance (dyn s·cm⁻⁵·m²)</td>
<td>583 ± 260</td>
</tr>
<tr>
<td>Pulmonary vascular resistance (dyn s·cm⁻⁵·m²)</td>
<td>312 ± 162</td>
</tr>
<tr>
<td>Cardiac index (litres · min⁻¹·m⁻²)</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>79 ± 14</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>92 ± 14</td>
</tr>
<tr>
<td>Systemic vascular resistance (dyn s·cm⁻⁵·m²)</td>
<td>2628 ± 1122</td>
</tr>
</tbody>
</table>

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AM-T and AM-m and the haemodynamic variables are shown in Figures 3 and 4. Plasma AM-m and AM-T levels were significantly correlated with the mean pulmonary artery pressure. Plasma AM-m and AM-T levels were also correlated with the total pulmonary vascular resistance.

### DISCUSSION

Previous studies have reported that plasma AM concentrations are increased in patients with primary pulmonary hypertension and secondary pulmonary hypertension due to mitral stenosis [9,10,19,20]. According to the
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Figure 4  Relationships between total pulmonary resistance (A) and plasma AM-T (B) levels in patients with mitral stenosis

Figure 4  Relationships between total pulmonary resistance and plasma AM-T (A) and AM-m (B) levels in patients with mitral stenosis

study of Kitamura et al. [12], plasma AM levels were reported previously as mean levels of AM-T, being the sum of AM-m and AM-Gly, because the radioimmunoassay system used polyclonal antibodies which could not distinguish between structures with or without a C-terminal amide. Kitamura et al. [12] measured plasma AM-m and AM-Gly levels using two kinds of radioimmunoassay systems after the extraction of large amounts of plasma. A one-step direct IRMA system for the measurement of AM-m and AM-T using different monoclonal antibodies has been developed recently [13,14], which enables us to measure AM-m and AM-T specifically in a small sample without the prior extraction of plasma. The IRMA for AM-m has no cross-reactivity with AM-Gly or other inactive metabolites of AM, and the IRMA for AM-T recognizes both AM-m and AM-Gly. In the present study, we showed using the IRMA for AM-T that plasma AM-T levels were increased in patients with mitral stenosis compared with normal controls, in agreement with previous reports [19,20]. We also showed that plasma AM-m and AM-Gly levels were increased significantly in patients with mitral stenosis compared with normal controls. In addition, plasma AM-m, AM-Gly and AM-T levels showed similar correlations with pulmonary arterial pressure and total pulmonary resistance. These findings indicate that the the production of AM-m and AM-Gly is regulated similarly under pathological conditions such as mitral stenosis.

AM-m represented approx. 13% of AM-T in the present study, suggesting that the major circulating form of AM is AM-Gly. This finding is consistent with a previous report [12]. The low AM-m/AM-T ratio in plasma may be explained by a low level of secretion of AM-m from tissues, or by a longer half-life of AM-Gly. Cultured endothelial cells and vascular smooth muscle cells produce large amounts of AM-m and small amounts of AM-Gly [2,3,21]. However, as AM acts as an autocrine and/or paracrine factor, AM-m produced in the tissues may be consumed almost entirely via receptor binding, and only small amounts of AM-m may be released into the circulation. In contrast, AM-Gly, an inactive form, cannot bind to the receptors, and therefore most of the AM-Gly produced may be released into the circulation. Furthermore, the low biological activity of AM-Gly suggests that the half-life of AM-Gly is longer than that of AM-m. This expected longer half-life of AM-Gly may, at least in part, be responsible for the low AM-m/AM-T ratio in human plasma.

In the present study, the plasma AM-m, AM-Gly and AM-T levels in the vein were similar to those in the pulmonary artery, whereas plasma AM-T levels were slightly lower in the left atrium than in the pulmonary artery. Plasma AM-m levels were markedly lower in the left atrium than in the pulmonary artery, although there were no differences in plasma AM-Gly levels between the left atrium and pulmonary artery. An autoradiographic study showed that an intravenous injection of 125I-AM was strongly taken up by the lung [22], suggesting that the lung has abundant binding sites for AM. In addition, a previous study reported that AM binding sites were highly concentrated in the lung [4]. Furthermore, a recent study revealed that AM receptor mRNA is highly expressed in human lung tissue [5]. Indeed, several studies have shown that plasma AM was partially metabolized in the pulmonary circulation of patients with primary and secondary pulmonary hypertension and ischaemic heart disease [10,20,23]. The present study demonstrated that this observed extraction of plasma AM-T in the pulmonary circulation is due mainly to the extraction of AM-m. A recent study by Hirayama et al. [21] showed that plasma AM-m levels were lower in the pulmonary capillary wedge portion than in the pulmonary artery portion in patients with ischaemic heart disease, which is in good agreement with the present study. As regards the action of AM, previous studies have demonstrated that AM preferentially and strongly dilates pulmonary vessels over systemic vessels in animals [6,7,24,25]. Furthermore, a recent study reported that the pulmonary vasodilatory activity of AM is much more potent than that of acetylcholine or ATP on a molar basis in patients with pulmonary hypertension [26]. Taken together, these findings suggest that: (1) AM-
m is produced in and secreted by the peripheral circulation; (2) the main clearance site for circulating AM-m is the lung; and (3) circulating AM-m may participate in the regulation of pulmonary vascular tone in patients with secondary pulmonary hypertension due to mitral stenosis.

In conclusion, the major molecular form of circulating AM is AM-Gly in normal subjects and in patients with mitral stenosis, and plasma AM-m levels increase in parallel with those of AM-Gly in mitral stenosis. AM-m is produced in the peripheral circulation, and the main site for clearance of circulating AM-m is the lung, which probably participates in the regulation of pulmonary vascular tone. Thus extracted AM in the pulmonary circulation may help to attenuate increased pulmonary vascular resistance in patients with mitral stenosis. Further studies are necessary to elucidate the exact role of plasma AM in pulmonary hypertension.

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